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(54) **Testing DNA samples for particular nucleotide sequences.**

(57)   A method for determining whether the DNA contained in
a test sample includes a particular nucleic acid sequence,
comprising the steps of:

a) extracting nucleic acids from the test sample,

b) digesting the extracted nucleic acids with restriction en-
zyme thereby to cleave the DNA or not at a particular se-
quence, depending on whether or not a restriction enzyme re-
cognition site is present in the sequence,

c) treating the product of (b) to form single stranded nucleic
acids,

d) contacting the single stranded nucleic acids produced in
(c) with first and second polynucleotide probes which are
complementary to respective first and second portions of said
sequence to be detected, the two portions being non-overlap-
ping and immediately adjacent to the restriction site in ques-
tion. The contact is performed under conditions favorable to
hybridization of said first and second probes to said sequence
to be detected, hybridization with both of said probes being
dependent upon whether in step (b) restriction did not occur,
said first probe being incorporated with a distinguishable label,

e) separating, by means of said second probe, (i) any result-
ing dual hybridization product comprising said sequence to be
detected hybridized to both said labeled first probe and said
second probe, from (ii) any unhybridized and singly-hybridized
labeled first probe, and

f) by means of said label detecting any of said separated dual
hybridization product which may be present.

The second probe is preferably fixed to a solid support
and can be used by mixing the first probe in solution with the
unknown and with the solid support carrying the second
probe, letting the mass stand under hybridizing conditions, se-
parating the solid support and determining the presence and
amount of label attached to the solid support.

Testing DNA Samples for Particular Nucleotide Sequences

The present invention relates to a process and
device for determining the presence or absence of
particular genetic conditions in samples of plant or
animal tissue or fluid, as evidenced by particular
5   alternative nucleic acid constitutions of cells in such
material. The condition need not be expressed clinically
for its presence or potential to be detected in this test.

Techniques are available for determining genetic
conditions such as sickle cell anemia by testing the DNA
10 in tissues, such as white blood cells, of a patient under
consideration. Such tests as presently constituted are
time-consuming, and require extreme skill in running the
test and interpreting the results. They can also be
costly, and then can be inaccurate.

15 It is an object of the present invention to provide
a simple, relatively inexpensive, and reliable high
resolution test for the presence or absence of particular
nucleic acid sequences in test DNA specimens.

These and other objects and advantages are realized
20 in accordance with the present invention pursuant to
which there is provided a method for determining whether
the nucleic acid in a test sample includes a particular
nucleic acid sequence, comprising the steps of:

a)   extracting the nucleic acids from a test
25 sample,

b)   digesting the extracted nucleic acids with a
restriction enzyme thereby to effect restriction or not
to effect restriction, depending on whether or not the
restriction enzyme recognition site is precisely present
30 in a sequence in the test DNA,

c)   treating the product of (b) to form single-
stranded nucleic acids,

d)   contacting the single-stranded nucleic acids
produced in (c) with first and second polynucleotide

5   probes which are complementary to respective first and
second portions of said sequence to be detected, the two
portions being non-overlapping and immediately adjacent
to the restriction site in question, and such contact
being performed under conditions favorable to hybridi-

10   zation of said first and second probes to said sequence
to be detected, hybridization with both of said probes
to a test molecule being dependent upon whether in step
(b) restriction did not occur, said first probe being
incorporated with a distinguishable label,

15   e)   separating, by means of said second probe,
(i) any resulting dual hybridization product comprising
said sequence to be detected hybridized to both said
labeled first probe and said second probe, from (ii)
any unhybridized and singly hybridized labeled first

20   probe, and

f)   by means of said label detecting any of said
separated dual hybridization product which may be present.

A suitable kit for running the test is also provided
comprising (i) first and second probes each including

25   nucleic acid sequences present in the nucleic acid of a
test sample, the first probe carrying a distinguishable
label and being soluble in a liquid in which the
determination will be run, the second probe being fixed
on a solid support, and (ii) a restriction enzyme which

30   will cleave the test sample or fail to depending on the

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presence or absence of a particular nucleic acid sequence at the point separating sequences complementary to the first and second probes, whereby a positive determination test sample (no cleavage at the point) is capable of

5   hybridizing with both the first and second probes so as thereby to affix the label to the solid support for subsequent reading. The presence of labelling material on the solid support is an index of the extent of dual hybridization.

10   The first and second probes are themselves formed in a special way. For example, a cloned ß-hemoglobin gene is digested by restriction enzymes which subdivide it into a number of fragments. In the case of detection of the sickle cell defect, the two of interest are a

15   340 bp (base pair) unit and a 201 bp unit. The fragments are separated from one another by subcloning, and one of them, the 340 bp unit fragment, is fixed to a solid support such as a nitrocellulose sheet or disc, con- stituting the fixed probe. The other fragment is labeled

20   either with a radioactive group, or a chemically detect- able group such as a modified base or a visually detect- able group such as one which fluoresces or has characte- ristic absorbance of ultraviolet or infrared light.

A key to the procedure, in the difference between

25   an affected and unaffected test sample, is that in one of them, upon treatment with a restriction enzyme, there will be fragments longer than each probe so as to be capable of hybridizing with both the fixed probe and the labeled probe whereas the other will not have such longer

30   units, so it will be incapable of hybridizing with both.

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The dual hybridization is the key to attaching the label
to the solid support via one test sample but not the
other, such support being what is ultimately analyzed
for the presence of label.

5   With sickle cell anemia as an example, a normal
hemoglobin gene can be used to make probes of 340 and
201 bp, and sickle cell anemia hemoglobin DNA, upon
digestion, will have a long fragment which covers the
two and can dually hybridize, while similarly treated
10   normal DNA will have no such fragment.

The manner of enzymatically digesting the DNA
material for producing the probes and/or treating the
test samples is known in the art, as are methods for
the various separations.

15   The invention has been particularly described with
reference to sickle cell anemia, but it is applicable as
well to any other genetic disorders whose presence can
be associated with a particular molecular alteration of
DNA. One need only find a particular restriction enzyme
20   which will degrade only one of an affected or unaffected
gene to form the fragments needed for making the probes
which can then distinguish between affected and unaffected
test samples pretreated in such manner as one or the
other will form a long fragment capable of dually hybri-
25   dizing with both probes.

The invention will be further described with
reference to the accompanying drawings, wherein:

Fig. 1 is a graphic representation of the hemoglobin
gene, the preparation of first and second probes and the
30   digestion products of normal and sickle cell DNA; and

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Fig. 2 is a flow sheet of the method of the
invention involving dual hybridization with first and
second probes and an unknown DNA.

Referring now more particularly to the drawings,
5   in Fig. 1, A is a map of the beginning of the gene encoding
ß-hemoglobin, together with flanking DNA that is not
expressed (the thin line to the left). The thick portion
defines DNA that is transcribed into RNA that is the
precursor of messenger RNA that is translated into
10   ß-hemoglobin, and this continues off the figure to the
right. Filled-in areas are those that actually encode
the amino acid sequence of the protein; the three clear
areas are, from left to right, an untranslated leader
sequence that is present in the mRNA, a short intervening
15   sequence, and a long intervening sequence. The two inter-
vening sequences are present in the RNA precursor to
globin mRNA, but are absent from the mRNA itself; they
are removed in intramolecular splicing events that are
part of the mRNA maturation process. Symbols above the
20   lines indicate restriction enzyme cleavage sites, and
the Dde I/Mst II site specifically absent from a sickle
cell gene is indicated. The 0.74 kb Alu I segment
represented in the cloned plasmid pSS737 is also indicated.

B shows examples of subcloned segments of the
25   ß-globin gene that can be used as probes in the dual
hybridization test. There is a Hinf I site 5 base pairs
upstream of the dimorphic DdeI/Mst II site, and this is
one end of a 0.34 kb Hinf I segment that has been cloned
via a plasmid vector (pBR322). The dimorphic Dde I site
30   itself is one end of a 0.20 kb Dde I segment that was

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subcloned via a phage vector (M13 MP8) that shares no
sequences with pBR. The reciprocal clonings have also
been done. In the drawing, flat lines are the cloned
human DNA segments, and jagged lines are adjacent
5   portions of vector sequence. The reason that adjacent
Dde I segments were not simply subcloned is that the
0.175 kb Dde I segment (see C below) is unnecessarily
short. The reason that adjacent Mst II segments (one of
which is 1.14 kb long; see C) were not subcloned is that
10   DNA treated with Mst II cannot be ligated.

C is a comparison of the relevant products of Dde
I or Mst II digestion of human DNA that contains the
normal ß-globin allele, with DNA that contains the sickle
cell allele. DNA from persons having the sickle cell
15   trait (+/s) will have both sets of DNA segments.

In Fig. 2 at the left and right one starts with
separation and detection probes produced as hereinabove
described. The denatured separation probe is attached to
a solid support. The detection probe is labeled and
20   denatured.

The unknown specimen is treated to extract its DNA
which is digested and denatured. Then the probes and
treated DNA are mixed under hybridization conditions.
The solid with any dually hybridized material attached
25   thereto is then assayed for the label.

The invention will be further described in the
following illustrative example wherein all parts are by
weight unless otherwise expressed.

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EXAMPLE

APPLICATION OF THE "DUAL HYBRIDIZATION" TEST TO SICKLE
CELL HEMOGLOBINEMIA

1. Required DNAs: For a simple example of the
utility of dual hybridization, one needs only the plasmid
pBR322βPst or the plasmid pSS737 (both obtained from Dr.
John Wilson of the Medical College of Georgia, and des-
cribed in Geever, et al (1981) Proc. National Academy
of Science U.S.A. 78, 5081-5085, or any other of the
several cloned human β-hemoglobin genes that are available,
together with segments subcloned thereform (see item 2,
below). For an example of its applicability to sickle
cell hemoglobinemia (and its extensibility to other
disorders that involve restriction site polymorphism),
one also needs DNA from +/+ and s/s individuals.

2.   Subcloning the probes: The plasmid pSS737 contains
a 0.74 kb Pst I segment of human DNA that includes the
β-hemoglobin gene (Fig. 1). The restriction site of
interest in the clones is a DdeI/Mst II site involving
codons 5-7 of the gene, as in the sickle cell allele there
is a single A to T change that alters the enzymes'
recognition/cleavage site (CTNAG for Dde I; CCTNAGG for
Mst II). The aim of subcloning is to dissect the cloned
wild-type gene at this point to make two probes having
adjacent (or, in general, nearly adjacent) and non-
overlapping sequences. One probe, designated the
separation probe, will be matrix-bound and single-
stranded, and will immobilize an unknown DNA by hybri-
dization. The other probe, designated the detector probe,

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will be labelled and also single-stranded, and will
hybridize with the unknown DNA in solution. The unknown
DNA will have been treated with the restriction enzymes
Dde I and/or Mst II, and the question is whether or not
5   the detector probe can become immobilized via a bridge
to the separation probe, the bridge being provided by
the unknown DNA. If the unknown DNA is wild-type with
respect to the ß-globin gene, Dde I or Mst II digestion
will obviate such a linkage. However, a sickle cell
10   allele will be insensitive to the enzymes at the
relevant site, so genomic DNA that is s/s or s/+ will
allow the immobilization of some detector probe (see
items 3 and 4 for the digestion of genomic DNA and the
labelling of detector probe).

15   The subcloning proceeds as follows: The plasmid
pSS737 is purified and one sample is digested to
completion with Dde I; another sample is digested with
Hinf I (see Fig. 1). The resulting DNA segments are
separated according to size by electrophoresis in a
20   preparative, low melting temperature agarose gel. The
gel is stained with ethidium bromide to allow visuali-
zation by UV transillumination, and DNA bands of 0.34 kb
from the Hinf I digest, and 0.20 kb from the Dde I
digest, are excised. Agarose in the gel slices is melted,
25   and DNA is purified by phenol extraction and ethanol
precipitation. The DNAs are redissolved in water. At this
point, the 0.34 kb Hinf I digestion products have the
structure

               5' ANTC......G
30   G......CTNA 5'

and the 0.20 kb

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Dde I digestion products have the structure

5' TNAGG....CC
            CC... GGANT 5'

It is desirable to clone the segments in such a way that
the respective Hinf I and Dde I/Mst II sites at the ter-
mini are retained, and the practical way to do this is to
blunt-end ligate the segments to a vector that has been
linearized with an enzyme that gives ends that, upon fu-
sion with a segment to be cloned, contribute to the resto-
ration of the enzyme cleavage site. In particular, Hinf I
segments can be inserted into pBR322 that has been diges-
ted with Cla I to restore the Hinf I cleavage sites; Dde I
(Mst II) segments can be inserted into pBR322 that has
been digested with Nae I, or into phage M 13 MP8 DNA that
has been digested with Sma I.

Cla I cleaves pBR322 once at the site

...AT CGAT....
....TAGC TA...

The filling-in of overlapping ends by the large (Klenow)
fragment of E. coli DNA polymerase produces

...ATCG   CGAT...
...TAGC   GCTA...

The filling-in of ends of the Hinf I segment ro be sub-
cloned produces

ANTC......GANT
TNAG......CTNA

and ligation of this with the vector results in a sequence,

...ATCGANTC......GANTCGAT...
...TAGCTNAG......CTNAGCTA...

that retains the Hinf I sites (overlines).

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    The 0.20 kb Dde I (Mst II) segment of the ß-globin
gene is to be ligated to the ends of a vector linearized
with an enzyme that gives the following ends:

                    ...CC   GG...
5   ...GG   CC...

One such enzyme is Sma I (CCC GGG), and this can be used
for cloning into the E. Coli phage M13 MP8, which has a
single Sma I site. Another such enzyme is Nae I (GCC GGC),
which cleaves pBR322 four times, at 401, at 1282, and at
10 two other sites in between. The plasmid is left intact
between 1282 and 401, and the only function disrupted is
the ability to confer tetracycline resistance. For the
cloning of the 201 pb Dde I segment, vector preparation
involves the digestion of pBR322 with Nae I and a
15 treatment of the products with alkaline phosphatase to
remove terminal phosphates (this discourages simple
vector circularization during subsequent ligation). The
large vector segment is purified by preparative gel
electrophoresis. To accomplish the blunt-end ligation,
20 the ends of the ß-globin Dde I segment are filled in to
make them flush. As suggested above, this is done by
treating the segments with the Kleno fragment of E. coli
DNA polymerase plus the four deoxyribonucleoside tri-
phosphates. A mixture of these segments and the Nae I
25 treated vector is incubated with T_4 DNA ligase plus ATP,
then is used to transform bacterial cells made competent
to take up naked DNA. The cells are plated on medium
containing an antibiotic, resistance to which is conferred
by plasmid that is taken up by cells (e.g., ampicillin
30 resistance in the case of pBR322).

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Colonies of individual transformants are screened
for plasmids of interest by growing some cells in a small
volume of liquid, then gently lysing the cells to release
plasmid DNA. The DNA is purified, treated with the

5   indicated restriction enzymes that give segments diagnostic
for the presence in the plasmid of the desired insert, then
the DNA is displayed according to size in an agarose gel.
Once appropriate recombinant plasmids have been identified,
colonies from which they originated are used to seed large

10   cultures from which several hundred micrograms of plasmid
are prepared. The colonies are also maintained on plates,
and stored frozen for later use.

In the specific case of the development of plasmid
probes for the detection of the sickle cell variant of the

15   β-hemoglobin gene, one plasmid will contain the 0.34 kb
Hinf I segment, and another contains the adjacent 0.20 kb
Dde I segment. Because there is at least the possibility
that in the dual hybridization scheme hybridization of
vectors could give false positive results, it is desirable

20   that the two probes be carried in unrelated vectors. This
is most cleanly done using an E. coli host/vector system
for one probe, and a B. subtilis host/vector system for
the other. Alternatively, one probe can be in an E. coli
plasmid such as pBR322, and the other can be in an E. coli

25   phage, such as M13.

3.   Immobilizing the separation probe and labelling
     the detector probe: . Plasmid containing the
separation probe, e.g., a subclone of the 0.34 kb Hinf
segment of pSS737 /Fig. 2) is treated with 0.1 m NaOH

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for 5 minutes, then chilled in ice. The sample is
neutralized with an equal volume of 0.1 N HCl, 0.9 M NaCl,
0.09 M Na citrate, then filtered under mild aspiration
through a nitrocellulose filter (e.g., BA 85 from

5   Schleicher and Schuell) that had been presoaked in
0.9 M NaCl, 0.09 M Na citrate (6 x SSC; 1 x Standard Saline
Citrate = 0.15 M NaCl, 0.015 M Na citrate). The filter is
then washed with 6 x SSC, then 70 % ethanol, and baked .
under vacuum at 80°C for a few hours, or with no vacuum

10 at 65°C overnight. At this point, the filter is ready for
hybridization procedures, but it can be stored dry for
many months.

The purpose of the alkali treatment of plasmid is
to denature the DNA. This renders it both capable of

15 binding to nitrocellulose (native DNA does not) and
available for subsequent hybridization with other single-
stranded DNA. Neutralization of the denatured DNA
solution with acid and the addition of salt (as 6 x SSC)
facilitate the binding of denatured DNA to nitrocellulose,

20 and the low temperature inhibits reannealing of the
plasmid while it is being loaded onto the nitrocellulose.
Baking of the filter finally immobilizes the DNA.

Labelling of the detector probe with, for example,
^{32}P can be accomplished in several ways, all of wich are

25 standard methods: The ends of a plasmid that has been
made linear by the action of a restriction endonuclease
can be labelled either by a reaction in which polynucleo-
tide kinase adds the terminal phosphate of γ^{32}P-ATP to the
5' end of DNA molecules; by the filling in of recessed 3'

30 ends that are generated by some restriction enzymes,

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through the use of the large (Klenow) fragment of E. coli DNA polymerase and α^{32}P-labelled deoxyribonucleoside triphosphates; or by the 3' terminal addition of labelled nucleotides through the action of terminal addition of

5 labelled nucleotides through the action of terminal deoxynucleotidyl transferase. More extensive labelling of a probe can be accomplished using phage T_4DNA polymerase, or by strand replacement DNA synthesis ("nick translation") using E. coli DNA polymerase,

10 labelled triphosphates, and plasmid that has been randomly nicked by deoxyribonuclease I to produce numerous synthesis primer sites. Other labelling schemes that do not involve radioactivity are also possible.

4. **Preparation of samples of unknown DNA:** For the

15 simplest example of the utility of dual hybridization, use can be made of the plasmid pBR322ßPst or the plasmid pSS737 (Fig. 2) as the unknown as long as it can be assured that the subcloned detector probe does not hybridize with the immobile selector subclone in the

20 absence of the "unknown" (for the purpose of the model, it does not matter that the detector can hybridize with the "unknown" through both globin segment homology and vector homology). Since the Pst and the SS737 segments are already in pBR322, the best way to construct the two

25 probes is to put the selector probe (the 0.20 kb Dde I segment, as discussed in (2) into a B. subtilis vector, or into the E. coli phage M13, and the detector probe into pBR322. To make pBR322 Pst a useful unknown, one

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aliquot is digested with Mst II and/or Dde I, and this represents DNA containing the wild-type globin gene; pSS737 can be treated with Dde I. A second aliquot of plasmid is linearized by an enzyme that cleaves
5 outside of the region in question, but ideally a segment is generated that has a length not too much greater than the 0.38 kb Dde I segment, nor the 1.34 kb Mst II segment that results from digestion of CNA containing the sickle cell allele of the globin gene. Such a mimic of sickle
10 cell DNA could result from the digestion of pBR322BPst with Alu I, giving a segment of 0.74 kb with the dimorphic Dde I/Mst II site in the middle. The same result obtains if pSS737 is treated with EcoRI, because SS737 has the identical Alu I segment, inserted into
15 the EcoRI site of pBR322 via synthetic EcoRI linkers.

For the example in which genomic DNA from s/s and +/+ is used, the DNA is most easily prepared from 10 to 50 ml of blood with separation from RBC's, lysing of white cells in high salt and detergent, extraction of
20 nucleic acids using phenol and an ethanol precipitation, as follows:

RAPID PREPARATION OF DNA

i).   To a sample of blood (or of amniotic fluid, urine or a dispersed biopsy) add 1/4 volume of 0.25 M Tris-HCl, pH 7.8, 0.5 M Na_2EDTA, 1 %
25   Nonidet P-40. Mix gently but thoroughly.

ii).   Collect the crude preparation of nuclei by low-speed centrifugation in a conical tube.

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Suspend nuclei in 0.5 ml 0.05 M Tris, 0.1 M
EDTA. Transfer to a 5 ml tube.

iii). Add 0.5 ml 4 M NaCl, 2 % Sarkosyl, and mix
vigorously and thoroughly. Add 1 ml phenol/
chloroform /1:1, phenol (satd. w/tris-EDTA)
and chloroform7, and emulsify. Continue vigorous
shaking for 5 minutes then centrifuge at high
speed.

iv). Collect the (upper) aqueous phase, leaving the
organic phase and the band of denatured protein
undisturbed. Transfer the solution of another
5 ml tube, then add 2.5 ml 95 % ethanol. Mix
thoroughly, then centrifuge at high speed.

v). Decant the supernatant, then add 3 ml 70 %
ethanol to tube. Mix vigorously (vortex) to
suspend the pellet, then centrifuge at high
speed. Decant the ethanol, then repeat the 70 %
ethanol wash. After centrifugation and decanta-
tion, add 3 ml 95 % ethanol, Mix, then centri-
fuge at high speed. Decant the ethanol, then
dry the tube.

vi). Dissolve the pellet in 0.1 ml restriction enzyme
digestion buffer, then add 0.01 ml restriction
enzyme. Incubate at 37°C for 1 hr. or more.

A ribonuclease plus amylase step can also be
included and, to ensure the purity of DNA, nucleic acids
can be centrifuged in a CsCl buoyant density equilibrium
gradient. The DNA is collected from gradient fractions

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by precipitation with three volumes of 70 % ethanol,
with the precipitate being washed with 70 % and 95 %
ethanol. The precipitate is dried, then DNA is dissolved
in TE (10 mM Tris.HCl, pH 7.6, 1 mM Na$_2$EDTA) or water.

5   In using genomic CNA as the unknown, as will be
the case in the best example and in the field, it is
important that restriction enzyme digestions reach or
closely approach completion. In particular, the DdeI/Mst II
site present in wild-type DNA, but absent in the sickle

10   cell gene, has to be cleaved in samples of DNA lacking
the sickle cell allele. Because Mst II that is commer-
cially available is not highly active, special care must
be taken to ensure that digestions are as complete as
possible. Such care can involve the use of an order of

15   magnitude more enzyme than is theoretically required. It
can also involve, in the special case of sickle cell,
the digestion of DNA with excesses of both Mst II and
Dde I (in this case, sickle cell DNA segments of interest
will be 0.38 kb long rather than 1.34 kb long, but this

20   does not matter very much). Increasing the length of
time of digestion should not be relied upon, since many
enzymes become inactive within an hour or less of
dilution into a digestion mixture.

5.   Hybridization: As the hybridization procedure

25   involves single-stranded DNAs, the nitrocellulose filter
upon which the selector probe is immobilized must be
pretreated so that the unknown DNA and the detector
probe do not bind to it indiscriminately. Such treatment

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commonly involves saturating available sites on the
filter with protein and polysaccharide in a mixture known
as Denhardt's solution (0.2 % each of bovine serum
albumin, ficoll and polyvinylpyrrolidone in water), in
5   which, along with some salt and buffer (e.g., 6 x SSC,
0.1 M Tris, pH 8), a filter is soaked for a few hours
at the temperature to be used for hybridization (e.g.,
65°C). The presoak solution is then replaced with
hybridization medium that includes denatured sample
10   (unknown) DNA and denatured detector probe, and DNA
annealing is allowed to proceed for a few hours. Two
representative hybridization conditions are: (i) 6 x SSC,
0.1 M Tris, pH 8, 65°C, the inclusion of Denhardt's
solution being optional; (ii) 4 x SSC, 40 % formamide,
15   40°C, $\pm$ Denhardt's solution. To minimize the volume of
liquid needed, and to prevent evaporation, hybridizations
are often done in plastic bags that have been pressed
flat and sealed.

       After hybridization, DNAs that have not been
20   faithfully base paired to the selector probe are washed
from the filter by a series of filter soakings in
solutions that demand extensive annealing for hybrid
stability to be maintained. For example, the filter is
soaked in two changes of a large volume of 0.2 x SSC at
25   65°C (at which low salt concentration poorly base paired
hybrids will dissociate), then in two changes of a large
volume of 0.2 x SSC at room temperature. The filter is
then dried by blotting on filter paper. If the detector
probe is labelled with ^{32}P, the filter is subjected to
30   autoradiography by overlaying the filter with a sheet of

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X-ray film which is developed after a time; alternatively,
filter discs or portions of a filter sheet can be assayed
for radioactivity by immersing them in water and measuring
Cerenkov radiation in a liquid scintillation spectrometer.

5   6.   Explanations of procedures in part 4:   With reference
to the foregoing procedures in Part 4, the purpose of the
addition of Tris, EDTA and Nonidet in (i) to a sample of
cells in fluid is to lyse cells, but not nuclei, and to
inhibit deoxyribonucleases while so doing. Tris is the
10   buffer tris(hydroxymethyl)aminomethane; Na_2EDTA is
disodium ethylenediamine tetraacetate, a chelator of
divalent cations that are required for DNase activity;
nonionic detergents such as NP-40 (or Triton X-100)
solubilize cell membranes but do not significantly affect
15   cell nuclei.
        The result of this step is that cells, particularly
RBC's, are disrupted, with virtually only nuclei re-
maining intact. The purpose is to condition the sample
so that nearly all cell material but nuclei will remain
20   in the supernatent during the centrifugation at low
speed in step (ii).
        The purpose of step (ii) is to concentrate nuclei
by low speed centrifugation (e.g., 1 kg for 5-10 min.),
and to separate them from the rest of cell material and
25   the fluid by simply pouring off the supernatant. The
nuclei are suspended in the Tris-EDTA of step (i)
without the detergent in order to transfer them to an
extraction tube for step (iii). The transfer can be

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avoided by using a centrifuge tube with a bottom which is open and threaded to accept a 5 ml cappable extraction tube which itself can sustain high-speed centrifugation in steps (iii) to (v).

5   If the pelleted nuclei are sticky, they can be resuspended by including the polyanions spermine and/or spermidine in the buffer (and optionally also in the 4x buffer used in step (i)).

       The purpose of step (iii) is to dissociate nucleo-
10   protein complexes with high salt, to denature protein with the ionic detergent Sarkosyl (Na lauryl sarcosinate, which has the advantage over SDS that it is soluble in high salt and in the cold), and to remove proteins from the solution by partitioning them into the organic
15   solvent phenol or precipitating them with phenol and chloroform.

       Upon centrifugation, the emulsion produced by shaking is separated into three phases: a lower organic phase, a band of denatured protein, and an upper aqueous
20   phase that contains nucleic acids and any carbohydrates (mainly glycogen) that may have accumulated in the cells.

       The purpose of step (iv) is to recover carefully the aqueous phase, then precipitate nucleic acids by making the solution about 70 % in ethanol. If collection
25   of the aqueous phase, by pipetting, is difficult due to viscosity created by the presence of high molecular weight DNA, vigorous shaking in step (iii) will suffi-
ciently reduce the size of DNA or it may be necessary to introduce some deliberate shearing of the DNA /as by
30   altering step (iii) so that suspended nuclei are treated

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first only with 2 M NaCl in a syringe, forced through a
27 ga. needle into the extraction tube to shear the DNA,
and then subjected to Sarkosyl and phenol/chloroform[7].

The purpose of the ethanol washes in step (v) is

5   to remove residual solvent and salts from the precipi-
tate of nucleic acids. After the 95 % ethanol wash, the
tube should be dried short of the point where the pellet
is completely dry. Damp DNA dissolves more readily than
dry DNA. One wants to remove practically all of the

10   ethanol so that it doesn't interfere in step (vi), but
not so much liquid that the DNA is totally dry. The tube
may be drained inverted for some minutes, then wiped
inside with a Q-tip except in the immediate vicinity of
the pellet.

15   The purpose of step (vi) is to dissolve the DNA in
a buffer that is suitable for the action of a restriction
endonuclease, then to digest the DNA with the enzyme.
Restriction enzyme buffers generally contain millimolar
amounts of Tris buffer, Mf^{++}, and mercaptoethanol, and

20   concentrations of salt as high as 0.15 M. Also, it is
sometimes desirable to include bovine serum albumin to
help stabilize the enzyme. Because salts, and the pre-
sence of Mg^{++} in particular, will inhibit the dissolution
of DNA, it is advantageous to dissolve the DNA in water

25   before adding a fractional volume of concentrated buffer/
salts. For example, the DNA pellet can be dissolved in
0.08 ml of H_2O, and to this can be added 0.01 ml of
10x buffer/salts and 0.01 ml of enzyme.

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It will be understood that the specification and
examples are illustrative but not limitative of the
present invention and that other embodiments within the
spirit and scope of the invention will suggest themselves
5   to those skilled in the art.

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CLAIMS:

1.   A method for determining whether the DNA contained in a test sample includes a particular nucleic acid sequence, characterized in that it comprises the steps of:

a) extracting nucleic acids from the test sample,

b) digesting the extracted nucleic acids with restriction enzyme thereby to cleave the DNA or not at a particular sequence, depending on whether or not a restriction enzyme recognition site is present in the sequence,

c) treating the product of (b) to form single-stranded nucleic acids,

d) contacting the single-stranded nucleic acids produced in (c) with first and second polynucleotide probes which are complementary to respective first and second portions of said sequence to be detected, the two portions being non-overlapping and immediately adjacent to the restriction site in question, such contact being performed under conditions favorable to hybridization of said first and second probes to said sequence to be detected, hybridization with both of said probes being dependent upon whether in step (b) restriction did not occur, said first probe being incorporated with a distinguishable label,

e) separating, by means of said second probe, (i) any resulting dual hybridization product comprising said sequence to be detected hybridized to both said labeled first probe and said second probe, from (ii) any unhybridized and single hybridized labeled first probe, and

f) by means of said label detecting any of said separated dual hybridization product which may be present.

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2.   A method according to claim 1, characterized
in that the second probe is in a form which enables the
hybridization product of the first probe and said sequen-
ce to be detected to be separated from unhybridized and
singly hybridized first probe.

3.   A method according to claim 2, characterized
in that the second probe is fixed to a solid support.

4.   A method according to any of claims 1 to 3,
characterized in that each of the probes is independently
produced by sub-cloning in a different host vector system.

5.   A method according to any of claims 1 to 4,
characterized in that the first and second probes are
obtained by digestion of a cloned nucleic acid containing
material identical with that of the test sample, digestion
producing two nucleic acid fragments, separating the two
fragments so that one becomes the second probe, and
applying a distinguishable label to that fragment which
becomes the first probe, application of the label being
effected either before or after digestion or separation.

6.   A method according to claim 5, characterized
in that the digestion is effected with restriction
enzymes.

7.   A method according to claim 5 or 6, characteri-
zed in that the first and second probes are produced by
digestion of a hemoglobin gene, the test sample being
the hemoglobin gene of a patient being tested for sickle
cell anemia.

8.   A method according to claim 7, characterized
in that the enzymatic digestion of the test sample is
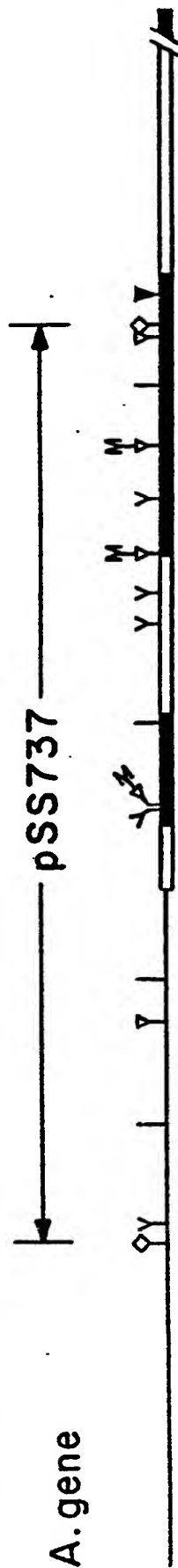such that it will leave sickle cell anemia nucleic acid

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of a length which can hybridize with both probes.

9.   A method according to claim 8, characterized
in that the first and second probes are produced by
digestion of a normal hemoglobin gene, hybridization
of the digested test sample to both indicating the
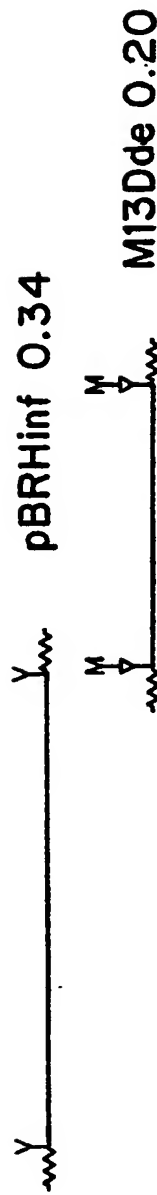presence therein of sickle cell anemia nucleic acid.

10.   A kit for determining whether the nucleic
acid contained in a test sample includes a particular
nucleic acid sequence, comprising (1) first and second
probes each including nucleic acid sequences present
in the nucleic acid of a test sample, the first probe
carrying a distinguishable label and being soluble in
a liquid in which the determination will be run, the
second probe being fixed on a solid support, and (2)
a restriction enzyme which will cleave the test sample
or fail to depending on the presence or absence of a
particular nucleic acid sequence, whereby a positive
determination test sample is capable of hybridizing with
both the first and second probes so as thereby to affix
the label to the solid support for subsequent reading.
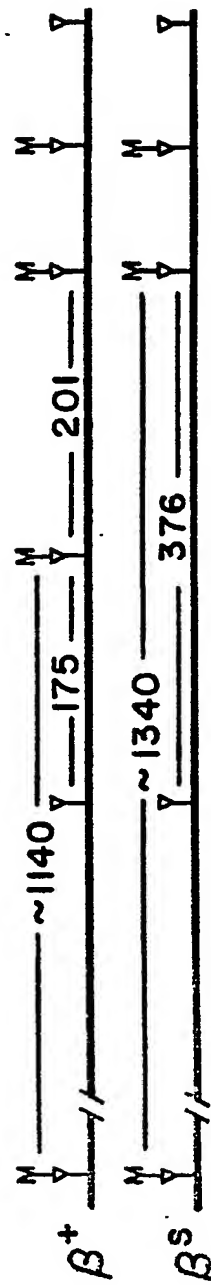
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FIG. 1

Sickle Cell Hemoglobinemia

A. gene

pSS737

dimorphic site

B. probes

pBRHinf 0.34

M13Dde 0.20

C. diagnostic digestion products

β⁺

β^s

~1140
175
201

~1340
376

AluI
BamHI
DdeI
HaeIII
HinfI
MstII

100bp

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FIG. 2

DUAL HYBRIDIZATION SCHEME

SEPARATION PROBE   SAMPLE FOR TESTING   DETECTION PROBE

DENATURATION   EXTRACTION OF DNA   LABELLING

                    UNKNOWN DNA

ATTACHMENT TO   RESTRICTION   DENATURATION
SOLID SUPPORT   ENZYME TREATMENT

IMMOBILIZED PROBE   DIGESTED DNA   LABELLED PROBE

                    DENATURATION

HYBRIDIZATION

POSITIVE   NEGATIVE